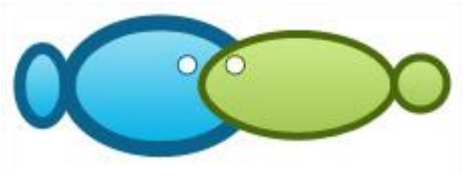

Genetic characteristics of *Macrobrachium lar* from Gane Timur, Halmahera Island, Indonesia, based on mitochondrial COI gene

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Abstract. *Macrobrachium lar* (Fabricius, 1978) is a prawn species belonging to the Palaemonidae family, which has a relatively wide distribution area in Indonesia, including Halmahera Island. In natural habitat, it serves as a prime target for fishermen, with fishing activities still largely unregulated. Several studies showed that unregulated fishing activities could lead to the excessive exploitation of resources. To overcome this challenge, various aspects necessary for managing aquatic resources need to be explored, including genetics. Therefore, this study aimed to examine genetic characteristics of *M. lar* prawn based on cytochrome oxidase subunit I (COI) gene. Prawn samples were collected from Nakalo River at Gane Timur, Halmahera Island, for analysis. A total of 10 grams prawn tissue were extracted, followed by DNA isolation using a Qiagen kit. The extraction results were then subjected to an amplification process with primers jgLCO and jgHCO using the PCR method. Subsequently, PCR product was visualized on 1% agarose gel and subjected to the sequencing process. Species validation results on National Center for Biotechnology Information (NCBI) website showed that the samples obtained were *M. lar* (identity 99.08%). Alignment results of the 441 bp COI gene sequence also revealed a varied range of genetic distance values. The intra-species genetic distance was 0.0110 (1.1%), the inter-species genetic distance was 0.0956 to 0.1949 (9.56 to 19.49%), while the genetic distance to the outgroup ranged from 0.2169 to 0.2316 (21.69 to 23.16%). In addition, the reconstruction of phylogenetic tree of the analyzed *Macrobrachium* species sequences formed two main clades, namely clades A and B. Clade A only consisted of *M. sintangense*, while clade B comprised *M. lar* (subclade B1) and *M. malayanum*, *M. naiyanetri* + *M. hirsutimanus* (subclade B2). Phylogenetic tree analysis confirmed that *M. lar* and some species of *Macrobrachium* were monophyletic.
Key Words: amphidromous species, COI, *Macrobrachium lar*, phylogenetic analysis.

Introduction. *Macrobrachium* is a genus of freshwater crustaceans in Palaemonidae family with a high level of diversity consisting of 240 identified species. Apart from inhabiting freshwater, *Macrobrachium* has also been reported to have the ability to thrive in brackish water (De Grave et al 2008; Wowor et al 2009). In addition, several species of the genus have been identified in various studies in Indonesia. These include *M. equidens*, *M. australe*, *M. latidactylus*, and *Macrobrachium lar* in rivers on Labobo Island, Sulawesi (Rahayu & Annawaty 2021), *M. scabriculum* in Batusuya River, Donggala (Dwiyanto et al 2017), as well as *M. lar*, *M. sintangense*, and *M. rosenbergii* in Beringin Kencana River, Kalimantan (a tributary of Barito River) (Fahlevi et al 2021). Other identified Indonesian species include *M. rosenbergii* in rivers on Ternate Island, Maluku (Samadan et al 2022, 2023) and *M. lar* in rivers in Manokwari, West Papua (Fadli et al 2018). Halmahera Island (Maluku) has been shown to be the distribution area of *M. rosenbergii*, *M. spinosum*, *M. idae*, *M. australe*, *M. lar*, *M. latidactylus*, *M. latimanus*, and *M. oenone* (Cai & Ng 2001). Among these species of *Macrobrachium*, *M. lar* is widely known to possess a relatively wide distribution area.

In line with previous studies, *M. lar* (Fabricius, 1978), commonly known as monkey river prawn, is an amphidromous species, where the larvae spend most of the

time in brackish water (Wowor et al 2009; Lal et al 2014; Sethi et al 2014; Castelin et al 2017). This species has the potential to be cultivated primarily due to its high economic value and relatively large sizes, with Lal et al (2012) also reporting its culture activity.

In natural habitat, it is a target for capture by local fishermen, including on Halmahera Island. However, continuous fishing activities without proper management efforts can cause high pressure on resources, specifically when fishing is carried out with environmentally unfriendly methods (Afara et al 2023; Bahtiar et al 2023a; Pratama et al 2023). In this context, studies on various aspects, such as biological (Bahtiar et al 2023b, c, d; Findra et al 2023), habitat (Findra et al 2016; Taula et al 2022), population (Findra et al 2020a; Bahtiar et al 2022a), reproduction (Bahtiar et al 2022b), and genetic (Indrayani et al 2021a, b), are necessary for managing aquatic resources.

According to several reports, genetic aspects play an essential role in the management of aquatic due to the association with the ability to adapt and develop (Findra et al 2017). Cytochrome oxidase subunit I (COI), a gene encoding mitochondrial DNA protein, is one of the genetic markers commonly used in analyzing genetic characteristics of animals, specifically those related to species and population studies (Solihin 1994; Findra et al 2020b; Hakim et al 2022). Despite the resourcefulness of the gene, there are no reports on the genetic aspects of *M. lar* in Gane Timur. Therefore, this study aims to examine the genetic characteristics of *M. lar* in Gane Timur.

Material and Method

Sample collection. *M. lar* samples were collected from Nakalo River at Maffa Village, Halmahera Island (Figure 1). Administratively, Maffa Village was included in Gane Timur District, South Halmahera Regency, North Maluku Province, Indonesia. Tissue samples were extracted from shrimp specimen, placed into a tube, and given 96% alcohol as a preservative. The collection of samples took place in September 2023.

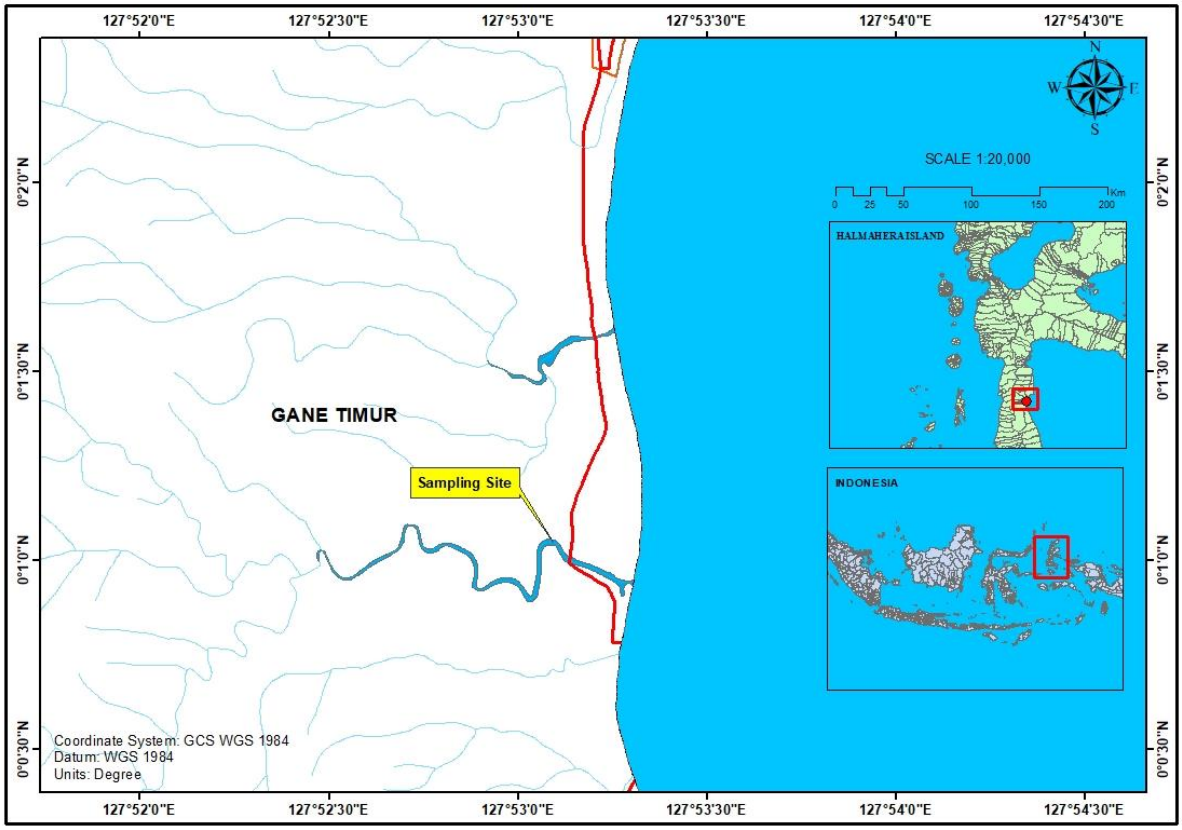

Figure 1. Sampling location at Gane Timur, Halmahera Island, Indonesia.

Total DNA isolation and extraction. A total of 10 grams prawn tissue samples were obtained for DNA extraction and isolation, and this method followed the Qiagen protocol.

The main stages of DNA extraction included cell lysis using 200 µL ATL Buffer and 20 µL Proteinase K, DNA binding by inserting the entire mixture into a 2 mL Dnaesy mini spin column tube and centrifuging at 800 rpm for 1 minute, followed by DNA washing with AW1 and AW2 buffer, and elution using 100 µL AE buffer (elution buffer).

Amplification and sequencing. The extraction results were analyzed for the next stage, which comprised amplification using polymerase chain reaction (PCR) following the BIONESIA laboratory protocol. The total reaction volume was 26 µL, comprising a mixture of 2 µL extracted DNA template, 1.25 µL each primer (forward and reverse), 9 µL ddH_2O, and 12.5 µL ready-mix. The primers used in the amplification process were jgLCO (5'-TIT CIA CIA AYC AYA ARG AYA TTG-3') and jgHCO (5'-TAI ACY TCI GGR TGI CCR AAR AA-3') (Geller et al 2013). Amplification was conducted using an Applied Biosystems™ 2720 Thermal Cycler machine, and PCR conditions included an initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 60 seconds. Denaturation to extension stages was repeated for 38 cycles, followed by a final extension stage at 72°C for 2 minutes. PCR product was visualized on a 1% Agarose gel using Nucleic Acid Gel Stain (GelRed®) staining, and samples emitting DNA bands proceeded to the sequencing process at PT. Genetika Science Jakarta.

Data analysis. The results of the sequencing process in the form of sequence data were edited and aligned, and the alignment used the ClustalW method using the MEGA 11 program (Tamura et al 2021). The data was matched with the database on National Center for Biotechnology Information (NCBI) website using the nucleotide Basic Local Alignment Search Tools (BLAST) method. The relationships were analyzed using phylogenetic tree, and this also confirmed BLAST results in identification up to the species level. In addition, phylogenetic tree was created using the Neighbor-Joining (NJ) method with bootstrap 1000 replication in MEGA 11, and the genetic distances were also analyzed to determine the genetic distance from other data using the p-distance method. Genetic distance analysis and phylogenetic trees used several sequences stored in Genbank, both ingroup and outgroup, i.e., *M. lar* (Acces. ON753707.1), *M. hirsutimanus* (Acces. MW845478.1), *M. malayanum* (Acces. MW845511.1), *M. naiyanetri* (Acces. MW845513.1), *M. sintangense* (Acces. MW845625.1), and *Penaeus monodon* (Acces. MF563564.1).

Results. Visualization of PCR products for *M. lar* prawn samples emitted single DNA bands, and the length of the sequence that was successfully amplified using primers jgLCO and jgHCO was 686 bp. The results of matching with data stored in GenBank through BLAST showed that the prawn sample was *M. lar* with an identity value of 99.08%. The results of BLAST could be seen in Table 1.

Table 1

BLAST results *of M. lar* sample from Gane Timur

Accession number	*Species name*	*Query cover (%)*	*E-value*	*Identity (%)*
ON753707.1	*Macrobrachium lar*	79	0.0	99.08
GU205068.1	*Macrobrachium lar*	69	0.0	99.16
GU205067.1	*Macrobrachium lar*	69	0.0	99.16
GU205066.1	*Macrobrachium lar*	69	0.0	99.16
MW845478.1	*Macrobrachium hirsutimanus*	97	0.0	85.52
MT235946.1	*Macrobrachium hirsutimanus*	97	0.0	85.52
MW845511.1	*Macrobrachium malayanum*	97	0.0	83.46
MW845513.1	*Macrobrachium naiyanetri*	97	0.0	84.35
MW845521.1	*Macrobrachium naiyanetri*	97	0.0	84.20
MW845625.1	*Macrobrachium sintangense*	98	0.0	83.41

Genetic distances and phylogenetic trees. The results of the genetic distance analysis of *M. lar* from the alignment of 441 bp of COI gene sequence using the p-distance method were 0.0110 for intra-species and 0.0956 to 0.1949 for inter-species. The results of this analysis could be seen in Table 2.

Table 2

Genetic distance among sequences of *M. lar* prawn from Gane Timur and other species from Genbank

No	*Species name*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
1	*Macrobrachium lar* (Gane Timur)							
2	*Macrobrachium lar* (ON753707.1)	0.0110						
3	*Macrobrachium hirsutimanus* (MW845478.1)	0.1507	0.1544					
4	*Macrobrachium malayanum* (MW845511.1)	0.1765	0.1728	0.1176				
5	*Macrobrachium naiyanetri* (MW845513.1)	0.1581	0.1599	0.0956	0.1121			
6	*Macrobrachium sintangense* (MW845625.1)	0.1710	0.1673	0.1783	0.1949	0.1710		
7	*Penaeus monodon* (MF563564.1)	0.2169	0.2169	0.2279	0.2408	0.2316	0.2279	

Phylogenetic tree of the relationship between *M. lar* and other shrimp was reconstructed using the Neighbor-Joining method with bootstrap 1000 replication. The analysis results showed that *M. lar* was in the same clade as *M. lar* from Genbank data with accession number ON753707.1 (Figure 2).

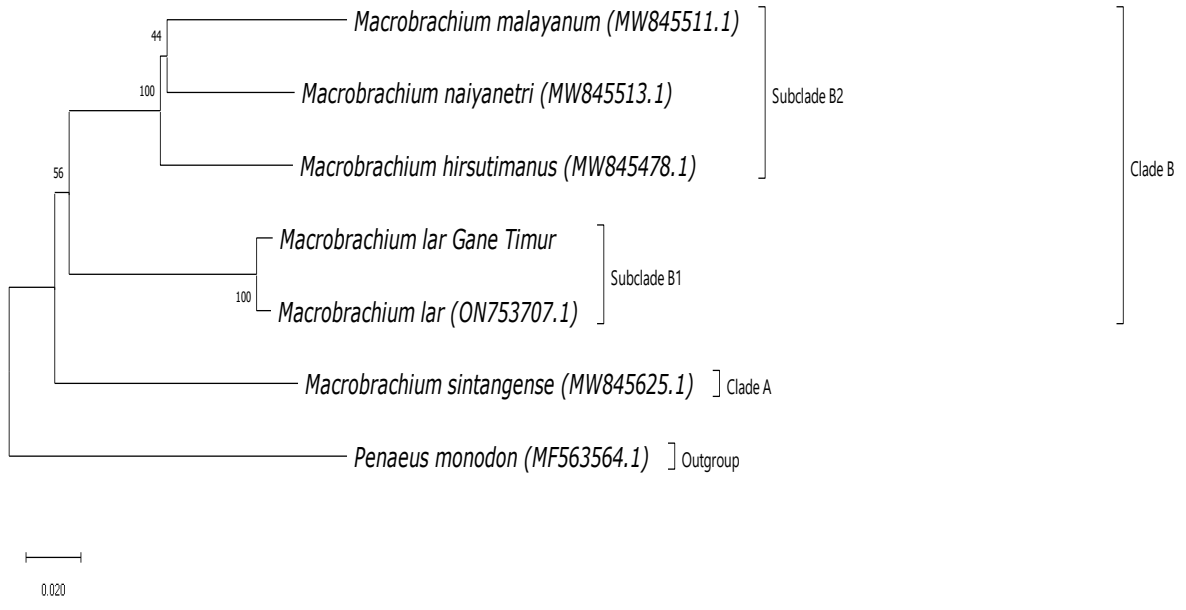

Figure 2. Phylogenetic tree of *Macrobrachium* sequences along 441 bp of the COI gene using the Neighbor-Joining method.

Discussion. The resulting partial COI gene sequence with the appearance of a single band showed that DNA extraction and amplification process was successful, and this was in line with the nucleotide BLAST results obtained, namely 99.08%, which was the same as the sequence stored in Genbank with accession number ON753707.1. In addition,

several other sequences, for instance Access. GU205068.1, GU205067.1, and GU205066.1 showed very high identity values (99.16%), and the identity value proved that the sample was *M. lar* with a very high level of similarity to the database stored in Genbank (close to 100%). The E value (Expected value) also showed a very low value, namely < 0.001 (Table 1), indicating that these sequences had significant similarities and did not occur by chance. Searching results for sequence ON753707.1 originate from Hong Kong (Chow et al 2022).

Alignment along the 441 bp COI gene sequence showed a varying range of genetic distance values (Table 2). The intra-species genetic distance of *M. lar* was 0.0110 (1.1%), the inter-species genetic distance was 0.0956 to 0.1949 (9.56 to 19.49%), while the genetic distance to the outgroup ranged from 0.2169 to 0.2316 (21.69 to 23.16%). This genetic distance showed the difference in proportion between 2 sequences in one population or group, meaning that the greater the genetic distance value, the higher the difference between the sequences. A genetic distance of 1.1% indicated that the difference between *M. lar* sample and ON753707.1 sequence was only 1.1%, and this could be ascertained that these 2 were the same species, as the genetic distance among *M. lar* sample sequence. Several other sequence analyses (ingroup) showed a value of 9.56 to 19.49%, and the difference was greater because it was a different species. The genetic distance between *Macrobrachium* species sequences (ingroup) and *P. monodon* (outgroup) showed an even greater value, namely between 21.69 to 23.16%, which indicated that the differences between these sequences were higher, and this was because both were different shrimp groups. COI gene variation of more than 4% was a close relative and a different species, and when the difference was less than 2 or 3% it was the same species (intra-species) (Hebert et al 2003; Ratnasingham & Hebert 2013).
The relationship among prawn sample and sequences from Genbank was increasingly visible in phylogenetic tree formed from reconstruction results using the Neighbor-Joining method (Figure 2). In general, *Macrobrachium* species sequences analyzed formed 2 main clades, for example, clades A and B, where clade B consisted of subclades B1 and B2. Clade A only consisted of *M. sintangense*, clade B consisted of *M. lar* (subclade B1) and *M. malayanum*, *M. naiyanetri* + *M. hirsutimanus* (subclade B2). The sample *M. lar* sequence joined *M. lar* sequence ON753707.1, and this further measured that the prawn sample from Gane Timur was indeed *M. lar* species. Meanwhile, several other sequences formed other clades. *M. malayanum*, *M. naiyanetri*, and *M. hirsutimanus* belonging to subclade B2 indicating that these 3 sequences were closely related to each other. The genetic distance between the 3 ranged from 9.56 to 17.65% (Table 2), with a within-mean group distance value of 10.85%. *M. sintangense* sequence (clade A) was separated from the other clades with between-group mean distance values of 16.9% and 18.1% for *M. sintangense* and subclade B2 (*M. malayanum* + *M. naiyanetri* + *M. hirsutimanus*) and *M. syntangense* and subclade B1 (*M. lar*). The between-group mean distance between the outgroup and subclade B2, B1, and clade A were 21.7%, 23.3%, and 22.8%, respectively. Judging from its relationship, *M. lar* was more closely related to prawn from subclade B1 and monophyletic which was visible in its genetic distance (Table 2), and when compared with *M. sintangense* (clade A), their relationship was more distant. However, phylogenetic tree also showed that *M. sintangense* was monophyletic with ancestors from *M. lar*, *M. malayanum*, *M. naiyanetri*, and *M. hirsutimanus*, and this meant that evolutionarily the existence of *M. sintangense* was older than other species. Phylogenetic tree reconstruction using COI, 16S, and 18S rRNA genetic markers also showed the same pattern, *M. malayanum,* and *M. naiyanetri* were in the same clade and sister taxa as *M. hirsutimanus*, and each was monophyletic (Siriwut et al 2020).

Conclusions. In conclusion, this study succeeded in revealing the characteristics of prawn sample from Gane Timur, Halmahera, based on the results of molecular analysis as *M. lar*. Genetic distance showed that this prawn was closely related to *M. malayanum*, *M. naiyanetri*, and *M. hirsutimanus*. In addition, phylogenetic tree reconstruction also showed the same thing, indicating that each was monophyletic.

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Conflict of interest. The authors declare that there is no conflict of interest.

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